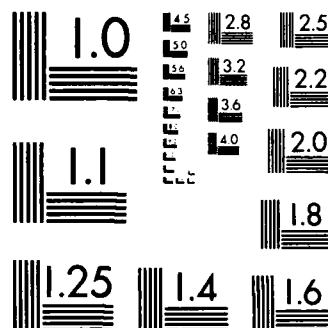


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MONOCLONAL ANTIBODIES THAT RECOGNIZE PROTEINS UNIQUE TO SOMATIC EMBRYOS OF *Daucus carota*

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ABSTRACT

We have developed a panel of monoclonal antibodies (MAbs) to several antigens of *Daucus carota* somatic embryos. We prepared over 2400 hybridoma cultures from splenocytes of Balb/c mice immunized with lyophilized somatic embryo cells. The MAbs were screened on embryo, callus cell, and chloroplast extracts using an automated sampling system and computer-assisted analyses. Most of the MAbs reacted with antigens common to embryo and callus cells, but MAbs from at least 5 hybridoma lines recognized single antigens unique to the somatic embryos. Other lines produced antibodies to chloroplast antigens, including the light-harvesting chlorophyll protein (LHCP).

INTRODUCTION

In the 27 years since Steward (1958) and Reinert (1959) first reported that carrot callus cells could differentiate into somatic embryos in vitro, cells of several other plant species have been shown to recapitulate in cell culture the stages of embryo development evident in seeds. However, little progress has been made since then toward understanding the mechanisms of the developmental change from callus cells to embryos. It is particularly difficult to get direct evidence for mechanisms because a variety of cell types and embryonic stages are observed after cultures are induced, and the changes in morphogenesis and differentiation occur asynchronously.

Comparison of one-dimensional electrophoretic patterns does not suffice for detecting expression of new gene products from induced cultures, but 2-dimensional electrophoretic techniques have shown unique proteins characteristic of embryonic morphogenesis (Sung and Okimoto, 1983; Choi and Sung, 1984). These proteins are of moderate to low abundance, and would not be likely to present a large number of antigenic determinants if they are part of a crude immunogen, nor would they be likely to present a large target for immunologic probes in situ.

One strategy for obtaining monoclonal antibodies to scarce proteins that can not be easily purified is to prepare and screen a sufficiently large number of hybridomas. Currently available culture techniques and automation make it easy to produce 1,000 or more hybridomas at a time. Selection of the desirable ones is the major problem. Hybridoma culture conditions require that the sampling and screening be completed in a "time window" of 48 to 96 hours, and it is often desirable to screen the cultures for differential response to multiple antigens. For large numbers of hybridomas, it is virtually impossible to evaluate such data objectively by visual inspection. In this paper we report selection of a panel of hybridomas to scarce tissue-specific antigens in carrot cultures, using an automated sampling system and novel computer programs for rapid identification of hybridoma antibodies specific for those antigens.

METHODS

Methods for culture of *Daucus carota* (Sung, 1976) and SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) were described previously. For enzyme immunoassay (EIA), 6 M urea extracts of the lyophilized tissues were prepared (____, personal communication), their protein content was determined by the method of Bradford (1976), Immulon II EIA plates (Dynatech) were coated with 1 µg of protein per well, and EIAs were performed essentially as described by Voller, et al. (1976). Protein immunoblot procedures were carried out as described by Towbin, et al. (1979), using peroxidase-conjugated goat anti-mouse globulin and 4-chloro-1-naphthol as the substrate (Hawkes, et al., 1982).

Male Balb/c mice were immunized with lyophilized carrot somatic embryo cells until the circulating antibody titer was >2000 by EIA. The mice were hyperimmunized 3 days prior to preparing the hybridomas. Splenocytes were fused with P3X63-AG8.653 myelomas essentially as described by Fazekas de St. Groth and Scheidegger (1980), and seeded at 2×10^4 cells/well in 134 96-well plates. At this seeding density, colonies that survived selection in aminopterin were monoclonal to >99% confidence. The hybridoma culture conditions will be detailed elsewhere (Smith, et al., in preparation).

Approximately 3,600 hybridoma colonies developed between the 8th and 21st days after fusion. Of these, 2,400 were sampled in two groups, using an automated system that directed and recorded the transfers on an Apple IIe computer (Karu, et al., 1985). Aliquots of each culture supernate were transferred to EIA plates coated with the callus, embryo, and chloroplast extracts, and EIAs were performed using alkaline phosphatase-conjugated goat anti-mouse Ig(G, M, A) globulin. Rates of color development were recorded from an EIA reader (Multiskan, Flow Laboratories) to a Commodore PET computer.

Figure 1 diagrams the general strategy of the screening procedure. The sampling and EIA data (approximately 12,000 data elements) were transferred to a Zilog Z-8000 computer and merged in a database from which histograms were created. Hybridomas having the best rates on one screening antigen, or the best ratio of rates on two antigens were selected and listed with respect to their location on the original culture plate. Using these systems, about 1,200 hybridoma supernates were harvested in 7 hr, and the data summaries could be examined the following day. Details of the procedures and computer programs will be published elsewhere (Smith, et al., and Neuschatz, et al., in preparation).

RESULTS

Figure 2 shows examples of the specificity of the screening EIA. Approximately 400 of the 2,400 hybridoma lines screened reacted strongly with at least one of the 3 plant extracts. Seventy-three of these showed specificity toward embryo extracts and negligible cross-reactivity with the callus and plastid extracts. When these hybridoma media were used to challenge immunoblots of embryo proteins separated by SDS-PAGE, roughly half failed to react, suggesting that these MAbs recognize denaturable epitopes. Twenty-nine of the MAbs bound to multiple bands resolved by 1-dimensional SDS-PAGE. These may be reacting with determinants shared by several size classes of protein. We have only begun to study the nature

carbohydrate modifications. Five of the MAbS reacted with unique bands in SDS-PAGE of embryo extracts (Figure 3). Several other MAbS showed specificity for chloroplast antigens. Figure 4 is an immunoblot challenged with two MAbS, one of which reacts with the LHCP bands in the chloroplast extract.

DISCUSSION

Monoclonal antibodies are powerful tools for detecting the differential expression and subcellular localization of antigens during somatic embryogenesis. Such MAbS would also be invaluable as probes of expression libraries of cloned plant genes. A number of developmental changes in plant cells are accompanied by post-translational modifications of proteins that are not per se expressed at a specific stage of development or differentiation, and MAbS specific for these modifications would clearly be useful.

In this study, we perceived a need for preparing large numbers of hybridomas, and the automated screening and computer-assisted evaluation methods enabled us to efficiently select and manage the most valuable hybridomas. The outcome leads us to seriously doubt that that we could have successfully obtained or recognized the 5 embryo-specific MAbS of Figure 2 by the more widely used small-scale hybridoma technology, but it is difficult to estimate and compare the likelihood of success in either method.

Experiments are in progress in our laboratory using these MAbS to monitor developmental changes and determine the subcellular sites and mechanisms of synthesis and modification that accompany these changes. We are attempting to assemble a panel of these MAbS to screen a cDNA expression library that we have created from somatic embryo mRNAs. We are hopeful that these studies will realize and demonstrate the usefulness of MAbS for studying the critical steps between callus cell growth and somatic embryogenesis.

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FIGURE LEGENDS

Figure 1. General strategy for rapid screening of large numbers of hybridomas. Statements not in boxes indicate manual procedures or decisions. Operations done by automation are indicated in rectangular boxes, and analyses performed on the mainframe computer are in boxes with rounded corners.

Figure 2. Reactions of hybridoma supernates on EIA plates coated with 6 M urea extracts (1 μ g protein/well) of lyophilized callus cells (A), somatic embryo cells (B), and mature leaf chloroplasts (C). Corresponding wells in each plate were challenged with aliquots of the same supernate. The arrows indicate MAb's that are callus-specific and embryo-specific, and one that cross-reacts with all 3 extracts.

Figure 3. SDS-PAGE profiles of callus (c) and embryo (e) extract proteins, and blot analyses of the immunoreactive components. (Panel A) Proteins separated in a 9% slab gel and stained with Coomassie Blue. (Panels B through E) Immunoperoxidase-stained protein blots challenged with (B) mouse immune serum against somatic embryo extract; (C) MAb 45G6, that reacts with shared antigens; (D) MAb 58H2 that reacts with an antigen common to callus and embryo cells; (E) MAb 21D7 that reacts with a single antigenic band unique to embryo extracts.

Figure 4. SDS-PAGE and protein immunoblot analysis of antigens recognized by MAb's 101A11 and 79F1. SDS-PAGE as described by Chua (1980) was used to separate proteins (50 μ g of extract protein per lane) from callus cells (c), embryos (e), and chloroplast membranes (p). Following electrophoresis, the proteins were transferred to nitrocellulose paper, and the blots were incubated with 0.5 ml of undiluted hybridoma culture medium. (Left panel) MAb 79F1, which reacts with chloroplast extract in the EIA, fails to react with the denatured extract. (Right panel) MAb 101A11 reacts with denatured proteins that were identified as LHCP by their apparent molecular weight and reaction with rabbit antisera to maize LHCP (provided by W. Taylor, Dept. of Genetics, U.C. Berkeley).

Automated sampling system

Identify wells with cultures growing in selective medium

Enter plate number and coordinates of wells to be sampled

Perform automated transfer of culture supernates into enzyme immunoassay (EIA) plates

EIA reader interfaced with personal computer

Perform EIAs; record results as absorbances or rates with 1 or more screening antigens

Transfer sampling & screening data to mainframe computer

Merge sampling & screening data in master file

For each screening antigen or combination, plot histogram of responses or ratio of responses

Use histogram to select response range for hybridomas to be expanded

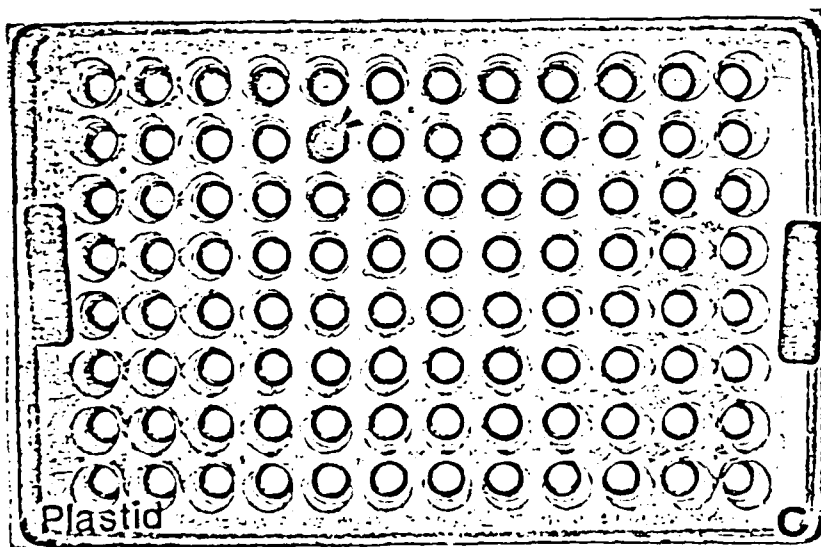
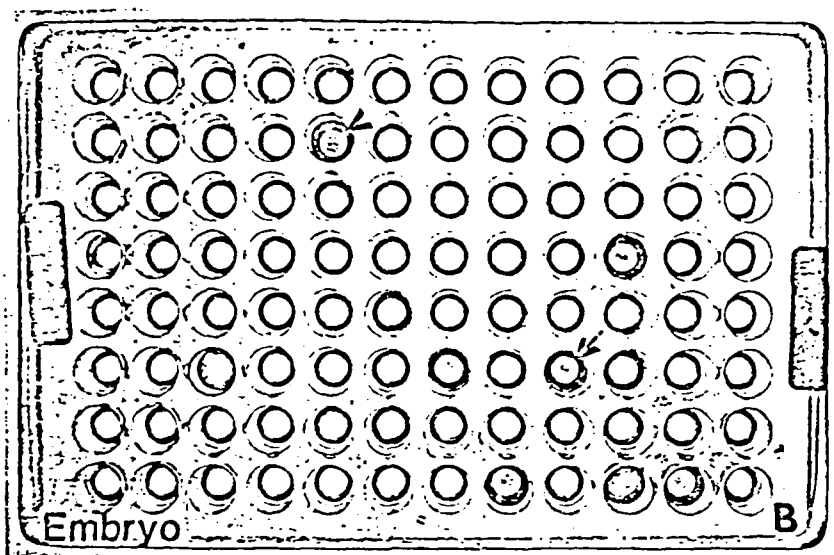
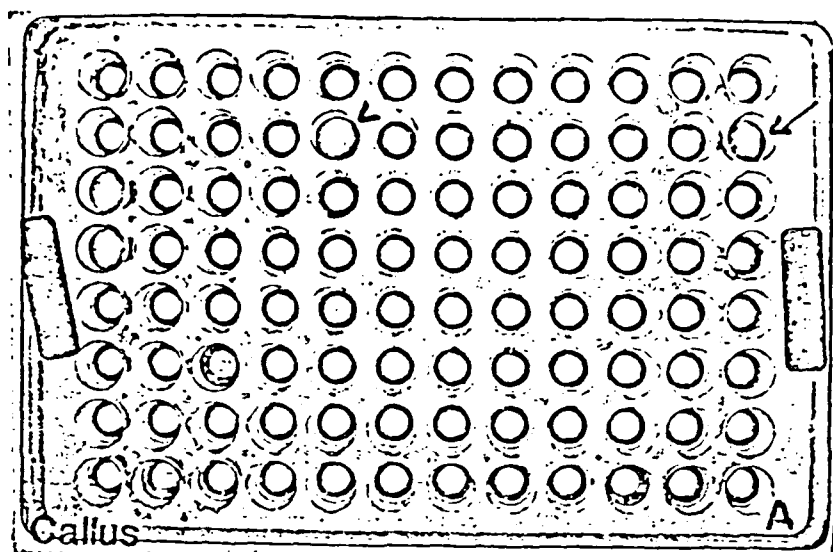
Repeat these steps to evaluate alternate choices

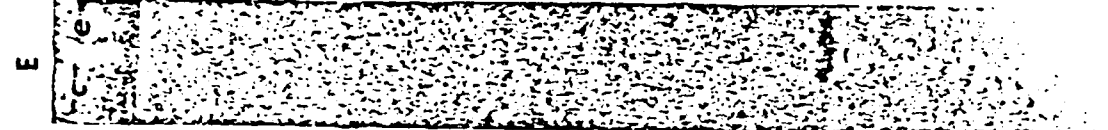
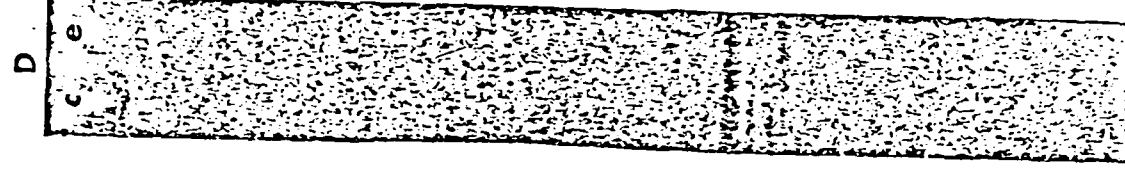
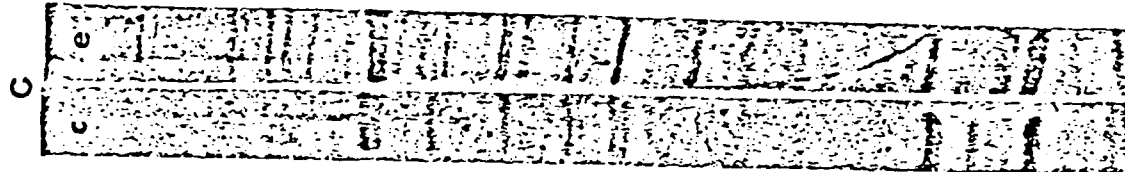
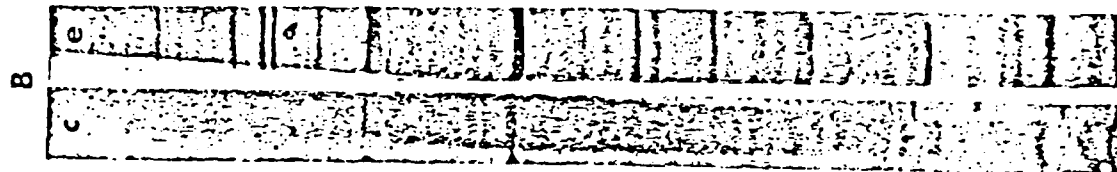
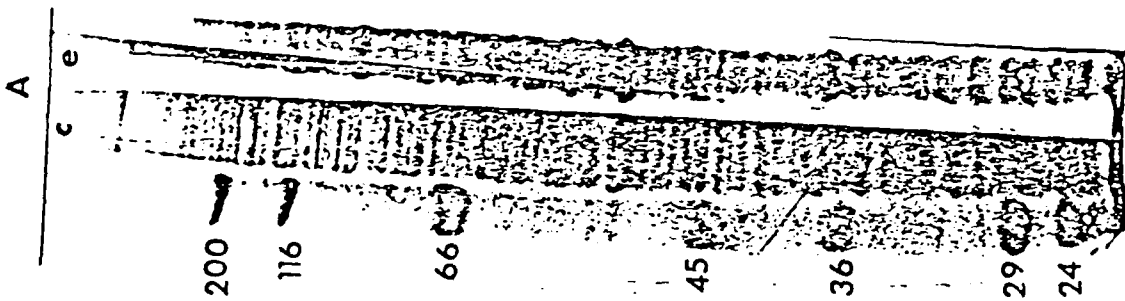
Automated sampling system

Expand selected hybridomas to larger culture wells

List the selected cultures by plate number, well coordinates, and response or response ratio

Use database and spreadsheet functions to store and evaluate subsequent assays of these cultures







LHCPs

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